LCK-phosphorylated human killer cell-inhibitory receptors recruit and activate phosphatidylinositol 3-kinase

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ABSTRACT HLA-specific killer cell inhibitory receptors (KIR) are thought to impede natural killer (NK) and T cell activation programs through recruitment of the SH2 domain-containing tyrosine phosphatases, SHP-1 and SHIP-2, to their cytoplasmic tails (CYT). To identify other SH2 domain-containing proteins that bind KIR CYT, we used the recently described yeast two-bait interaction trap and a modified version of this system, both of which permit tyrosine phosphorylation of bait proteins. Using these systems, we show that KIR CYT, once phosphorylated by the src-family tyrosine kinase LCK, additionally bind the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase. Furthermore, we show that in an NK cell line, NK3.3, cross-linking of KIR results in recruitment of p85 to KIR and activation of PI 3-kinase lipid kinase activity. One consequence of KIR coupling to PI 3-kinase is downstream activation of the antiapoptotic protein kinase AKT. Therefore, in addition to providing negative signals, KIR may also contribute positive signals for NK and T cell growth and/or survival.
Hitherto, PI 3-kinase and AKT have been primarily implicated in cell growth and/or survival (18, 19). Therefore, KIR may be involved in positive as well as negative signaling on NK and T cells.

**MATERIALS AND METHODS**

The Yeast Two-Bait Interaction Trap and Modified Two-Bait Interaction Trap Systems. Vectors used for the yeast two-bait interaction trap and modified two-bait interaction trap were as described (17). Briefly, pCWXX200 was used to direct expression of TetR-bait1, pEG202 of LexA (two-bait system) or TetR (modified two-bait system) fused to LCK, and pJG4–5 of a prey protein [putative interactor protein fused to a transcriptional activator (TA)]. All cDNA inserts were generated by using PCR and were verified by sequencing. Baits used included the CYT of human KIR–CL42, CL6, CL39, NKAT3, NKAT4, KIR103AS (3) and CD28 (20). Y-to-F substitution mutations of CL6 CYT in pCWXX200 were produced with the use of a QuickChange site-directed mutagenesis kit (Stratagene). The LCK kinase domain alone (21) was subcloned either directly into pEG202 (LexA-LCK) or first into pCWXX200 before being swapped into pEG202 along with TetR (TetR-LCK). An additional control construct included pEG202 encoding TetR alone. Known interactor proteins included both SH2 domains of human SHP-2 (22) and the complete coding regions of human Grb-2 (23) and p85α (24).

To assess interaction of bait proteins with known interactors, CWXY2 yeast were triply transformed with appropriate test or control pCWXX200, pEG202, and pJG4–5-based plasmids, and transformants were selected by culture on Glu+/LHW dropout plates at 30°C (25). Twenty to thirty colonies were then streaked onto Gal+ Raff+/-LHWUra dropout plates containing 150 ng/ml 6-azauracil, and yeast growth was scored after an additional 2–3 days of incubation. For library screening, CWXY2 yeast were transformed with pCWXX200-CL6 CYT and pEG202-LCK (LexA fusion) and selected on Glu+/-LHW dropout plates. Twenty to thirty colonies were pooled, grown, and transformed with a HeLa cDNA library contained in pJG4–5 (26). Yeast were grown in liquid Gal+ Raff+/-LHW dropout medium for 4 h before culture for 5 days on Gal+ Raff+/-LHWUra dropout plates containing 150 ng/ml 6-azauracil. Library plasmids were rescued from growing colonies and retransformed into CWXY2 yeast along with pCWXX200 and pEG202, pCWXX200-CL6 CYT and pEG202, or pCWXX200-CL6 CYT and pEG202-LCK. Yeast were selected on Glu+/-LHW dropout plates and then examined for growth on Gal+ Raff+/-LHWUra dropout plates as indicated above. Sequencing of pJG4–5 inserts was performed for those rescued pJG4–5 plasmids whose protein products permitted Ura-independent growth contingent on the coexpression of CL6 CYT plus LCK.

**Yeast Protein Expression.** Twenty to thirty yeast colonies from Ura+ dropout plates were pooled and grown overnight in liquid Gal+ Raff+/-Ura+ dropout medium. Whole yeast cell lysates were prepared as described (25), run on 10% SDS/PAGE gels, and transferred to poly(vinylidene difluoride) (PVDF) membranes (Dupont/NEN). Tyrosine-phosphorylated yeast proteins were detected by probing membranes with horseradish peroxidase (HRP)-conjugated RC-20 antibody (Transduction Laboratories, Lexington, KY) as described (27). To detect expression of TetR fusion proteins or pJG4–5 encoded proteins, membranes were stripped and probed with a rabbit polyclonal anti-TetR antisemur or 12CA5 anti-hemagglutinin (HA)mouse mAb (Boehringer Mannheim) followed by protein A-HRP (Zymed) or goat anti-mouse Ig (GM)-HRP (Sigma), respectively, again as described (27).

**KIR-p85α Association in NK Cells.** The NK cell line NK3.3 (28) was maintained in RPMI medium 1640 supplemented with 15% fetal calf serum (FCS), 15% Lymphocult (Biostat Diagnostics, Danville, NJ), 2 mM L-glutamine, and antibiotics. For coimmunoprecipitation experiments performed on exponentially growing cells, cells (50 × 10^6/data point) were lysed by resuspension in 600 μl of 1× Nonidet P-40 lysis buffer and murine mAb, either W6/32 (anti-class I HMC, IgG) (American Type Culture Collection) or GL183 (anti-KIR, IgG) (Immunotech, Westbrook, ME), were incubated with lysates for 15 min on ice (final concentration 5 μg/ml). For coimmunoprecipitation experiments in which the effect of KIR cross-linking was examined, rested cells (27) (40 × 10^6/data point) were resuspended in 300 μl of PBS with 5 μg/ml of GL183 and incubated on ice for 1 h. GAM (Sigma) was then added to samples (final concentration 20 μg/ml), and samples were transferred to 37°C for various times as indicated. Cells were lysed by the addition of an equal volume of 2× Nonidet P-40 lysis buffer.

Cell debris was removed from lysates by centrifugation, and lysates were precleared by rotation with 50 μl of packed Sephadex G-50 beads (Sigma) for 2 h. Antibodies were then rescued from lysates by rotation with 10 μl of packed protein-G agarose beads (Santa Cruz Biotechnology) for 1 h. Beads were washed five times in 1× Nonidet P-40 lysis buffer and boiled for 5 min in 1× SDS sample buffer. Eluted proteins were then run on 10% reducing SDS/PAGE gels and transferred to PVDF membranes. PI 3-kinase was detected by probing membranes with a rabbit anti-p85α antisemur (Upstate Biotechnology, Lake Placid, NY) and protein A-HRP (27).

**Kinase Assays.** Rested NK3.3 cells were stimulated with GL183 mAb and lysed as indicated above. In addition, in AKT kinase experiments, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and Ionomycin, and the effect of the PI 3-kinase inhibitors, wortmannin and LY294002, was examined at concentrations that specifically inhibit PI 3-kinase activity (27). One micromolar of anti-p85α antisemur or 2 μg of a rabbit anti-AKT antisemur (Upstate Biotechnology) were incubated with precleared lysates for 15 min on ice, and either p85α or AKT was precipitated by rotation of lysates with protein G-agarose beads for an additional 1 h. Lipid kinase activity of precipitated PI 3-kinase was assayed as described previously (29) but unlabeled PI 4-phosphate (PI-4P) was used as a substrate. Protein kinase activity of AKT was measured as described (30) by using histone 2B as a substrate, Radioactive 32P-labeled phosphatidylinositol 3,4-biphosphate (PI-3,4P2) or histone 2B was detected by autoradiography. Western blotting was used to verify that equal quantities of kinase were analyzed in all samples used for kinase assays.

**RESULTS**

Use of the Yeast Two-Bait Interaction Trap for the Detection of Phosphotyrosine-Dependent Protein–Protein Interactions. The yeast two-bait interaction trap has been described recently (17). In the two-bait system, interaction of a prey protein with both a bait1 fused to the DNA binding domain of TetR and a bait2 fused to the DNA binding domain of LexA is determined. Here, we used this system to study phosphotyrosine-dependent protein–protein interactions. For this purpose, prey protein interaction with a bait2 was not assessed. Rather, bait1 was substituted by a PTK. In such a system, one outcome that might be envisaged is that the PTK would phosphorylate bait1, thereby promoting an interaction of bait, with a prey protein. In this scenario, the recruited prey would then drive URA3 expression from an integrated TetOp-URA3 reporter gene that would confer on yeast an ability to grow in the absence of exogenous uracil (Fig. 1A).

We also considered a modified version of this system for these studies (Fig. 1B). In the standard two-bait system the PTK is fused to the DNA binding domain of LexA, which is functionally inactive in this context. However, in the modified system the PTK, like bait1 (hereafter referred to as bait), is...
SHP-2 is seen to bind almost exclusively to Y282 in this system. LCK phosphorylates both CL6 CYT ITIMs. Despite this, present in CL6 CYT, Y282 and Y312 (see Fig. 3) foundly reduced when either of the two ITIM-based tyrosines is strongly phosphorylated by TetR-LCK in the modified system. As shown in Fig. 2, Western blots (also as expected, yeast growth is more rapid in the modified two-bait system. As expected, TetR-LCK much more effectively phosphorylates the bait protein than LexA-LCK, thus allowing interaction with a prey protein and subsequent URA3 gene transcription. In the standard system the PTK is fused to LexA and is not targeted within the cell. In the modified system the PTK is also fused to TetR, resulting in juxtaposition of the PTK to bait.

fused to the DNA binding domain of TetR. Therefore, in the modified system, we would expect some of the TetOp to be occupied by PTK, thus increasing the local concentration of PTK in the vicinity of the bait protein. In turn, this might lead to more effective phosphorylation of the bait protein, which could increase assay sensitivity (31). Alternatively, the standard system may be a more appropriate system for the screening of libraries of prey proteins, where increased background noise that might otherwise result from the binding of prey proteins to the PTK itself would not be desirable.

**LCK-Mediated Physical Interaction Between a KIR CYT and the PTPase SHP-2 Demonstrated in Yeast.** To validate the above-described application of the two-bait interaction trap, we attempted to reconstitute in yeast an interaction between a nominal p58 KIR CYT, CL6 CYT, and the SH2 domains of the PTPase SHP-2. For phosphorylation of CL6 CYT we used the src-family PTK, LCK, because LCK has been demonstrated to phosphorylate KIR CYT in T and NK cells (13). However, rather than full length LCK, which was found to be toxic in yeast, we used the LCK kinase domain only. As shown in Fig. 2A, yeast transformed with TetR-CL6 CYT, TA-SHP-2, and either LexA-LCK or TetR-LCK acquire the ability to grow on uracil dropout plates. By contrast, yeast transformed with only one of these hybrids or with each of the three different pairwise combinations of these hybrids fail to grow in the absence of exogenous uracil. These results are consistent with the notion that LCK phosphorylates CL6 CYT on tyrosine residues, resulting in the binding of SHP-2.

Tyrosine phosphorylation of CL6 CYT was examined in the modified two-bait system. As expected, TetR-LCK much more effectively phosphorylates bait proteins than LexA-LCK, thus allowing visualization of bait tyrosine phosphorylation on Western blots (also as expected, yeast growth is more rapid in the modified system). As shown in Fig. 2B, wild-type CL6 CYT is strongly phosphorylated by TetR-LCK in the modified system. Furthermore, phosphorylation of CL6 CYT is profoundly reduced when either of the two ITIM-based tyrosines present in CL6 CYT, Y282 and Y312 (see Fig. 3A), are mutated to phenylalanine (Y282F and Y312F), and is completely eliminated when both of these tyrosines are mutated to phenylalanine (Y282F/Y312F). These results show that TetR-LCK phosphorylates both CL6 CYT ITIMs. Despite this, SHP-2 is seen to bind almost exclusively to Y282 in this system (Fig. 2C). Hence, the Y282F CL6 CYT mutant fails to support SHP-2-mediated uracil-independent yeast growth. In contrast, the Y312F CL6 CYT mutant supports yeast growth comparable to that seen with wild-type CL6 CYT. This same dependency on Y282 for the SHP-2 interaction was also seen when LexA-LCK was used as the kinase (Fig. 2C).

**Identification of the p85α Regulatory Subunit of PI 3-Kinase as an Additional Signaling Moiety That Binds Tyrosine-Phosphorylated KIR CYT.** To identify novel binding partners of tyrosine-phosphorylated KIR CYT, we transformed yeast with TetR-CL6 CYT, LexA-LCK, and a TA-HeLa cDNA library contained in pJG4-5. Of a total of $4 \times 10^8$ screened colonies, 18 that grew in the absence of exogenous uracil were identified. We determined that pJG4-5 plasmids rescued from...
representative KIR CYT from each of the six KIR groups that contain CYT tyrosine residues for their ability to bind p85α (Fig. 3A). As shown, as well as CL6 CYT, the p140 NKT4 CYT bound p85α. In addition, p58 CL42 and p70 NKT3 CYT bound p85α, albeit weakly compared with CL6 and NKT4 CYT (stronger interaction was detected by using TetR-LCK as the kinase). In contrast, p50 CL39 CYT and the KIR103AS CYT did not bind p85α at all. With the exception of CL39 CYT, which contains only a single tyrosine residue in a truncated ITIM motif (Fig. 3A), all KIR CYT bound SHP-2. This result indicates that amongst these KIR tails LCK phosphophorylates Y282 or the equivalent of Y282 comparably, and that the extent to which these tails bind p85α relates directly to their ability to function as ligands for the SH2 domains of p85α. None of the KIR CYT bound the SH2 domain-containing adaptor protein Grb-2. Conversely, the human CD28 CYT bound Grb-2 and p85α, as has been reported previously for this costimulatory receptor in T cells (32), but did not bind SHP-2. In all examples in which binding was demonstrable, this binding depended on the expression of LexA-LCK (Fig. 3A).

**Physical and Functional Interactions Between KIR and PI 3-Kinase in NK Cells.** We examined whether p85α is physically associated with KIR in NK cells, and for this purpose the human NK cell line NK3.3 was utilized. KIR were precipitated from exponentially growing NK3.3 by using the GL183 mAb, which recognizes subsets of KIR including those that possess the CL6-type CYT (1). As shown in Fig. 4A, p85α could be detected in KIR immunoprecipitates but not in control class I MHC immunoprecipitates from exponentially growing NK3.3.

To determine whether cross-linking of KIR on NK cells leads to the recruitment of p85α to KIR CYT, we first rested NK3.3 cells to reduce background KIR phosphorylation, which likely accounts for the constitutive KIR–p85α association seen in exponentially growing cells. After cell resting, KIR were either cross-linked or not cross-linked for various periods with GL183 plus GAM before precipitation of KIR from all samples (Fig. 4B). As expected, p85α was not found in KIR immunoprecipitates from rested NK3.3 cells (time point 0). However, p85α was readily detected in KIR immunoprecipitates from stimulated NK3.3 cells. This induced association between KIR and p85α was maximal at 10 min and had declined by 30 min poststimulation.

We examined in parallel whether KIR cross-linking on NK3.3 cells induces an increase in the lipid kinase activity of PI 3-kinase. In these experiments, p85α was directly immunoprecipitated from rested NK3.3 cells or with GL183 plus GAM and tested for its ability to transfer ^32^P to PI 4-phosphate in vitro kinase reactions (Fig. 4C). As shown, KIR cross-linking induced an increase in the lipid kinase activity of PI 3-kinase, which peaked at 10 min and returned to almost resting levels by 30 min poststimulation. Therefore, this activation of PI 3-kinase temporally parallels the recruitment of p85α to KIR CYT.

**KIR-Induced AKT Activation.** PI 3-kinase is upstream of several cellular-signaling intermediates including the antiapoptotic serine/threonine kinase AKT (18, 19). To examine whether KIR cross-linking also results in activation of AKT, rested NK3.3 cells were stimulated or not with GL183 plus GAM for 10 min, AKT was precipitated from lysates, and AKT kinase activity was determined by the ability to transfer ^32^P to a histone 2B substrate. Fig. 5 shows that cross-linking of GL183 reactive KIR on NK3.3 indeed stimulated an increase in AKT kinase activity. Furthermore, this activation was potentiated when PMA and ionomycin were used as costimuli at concentrations that by themselves did not affect AKT kinase activity. Illustrating a required role for PI 3-kinase enzymatic activity in KIR-mediated AKT activation, the specific PI 3-kinase inhibitors wortmannin and LY294002 were able to block AKT activity.
Fig. 4. KIR recruitment and activation of PI 3-kinase in NK cells. (A) Exponentially growing NK3.3 cells were lysed, and either class I MHC or KIR were immunoprecipitated by using the W6/32 or GL183 mAb, respectively. Coimmunoprecipitated p85α was detected by Western blotting. (B) Rested NK3.3 cells were stimulated with GL183 plus GAM for the indicated times at 37°C. Cells were lysed, and GL183-reactive KIR were immunoprecipitated from all samples. Coimmunoprecipitated p85α was detected as in (A). The position of p85α in an unfractionated NK3.3 lysate is shown at right. (C) Rested NK3.3 cells were stimulated with GL183 plus GAM for various times as in (B). Cells were lysed, p85α was immunoprecipitated, and immunoprecipitates were tested for their ability to transfer 32P to PI 4-phosphate. The position of radioactive phosphatidylinositol 3,4-biphosphate is shown.

**DISCUSSION**

Yeast two-hybrid technology represents a powerful technology that has been used to explore protein–protein interactions in vivo. However, the original technology was limited by its inability to allow the study of interactions involving more than two molecular components. Recently, to overcome this problem, we and others have developed yeast systems that allow the introduction of a third molecular entity (17, 33, 34). Herein, we describe the application of one such system, the yeast two-bait interaction trap, to the study of protein–protein interactions dependent on tyrosine phosphorylation.

We used this system to further explore signaling pathways initiated by KIR. KIR signaling is noted to involve LCK activation, tyrosine phosphorylation of CYT ITIM motifs, and recruitment and activation of SHP-1 (14). Additionally, because tyrosine-phosphorylated KIR ITIM peptides bind SHP-2, it has been suggested that this PT-Pase also becomes recruited to KIR during KIR signaling (35). We confirm here that after phosphorylation by LCK, SHP-2 is recruited to all types of tested KIR CYT that contain complete ITIM sequences. Also, we show that for one nominal p58 KIR CYT, CL6 CYT, SHP-2 is predominantly recruited to the membrane-proximal ITIM, even though the two ITIMs that are present in this tail are comparably phosphorylated by LCK. Likewise, SHP-1 is predominantly recruited to the membrane-proximal ITIM of p58 KIR CYT (14). Moreover, our data is in agreement with the finding that SHP-2 binds a membrane-proximal tyrosine-phosphorylated ITIM peptide with higher affinity than a membrane-distal tyrosine-phosphorylated ITIM peptide, both derived from a p58 receptor (35).

However, rather than SHP-2 binding, the main finding of the current studies is that LCK-phosphorylated KIR CYT bind the p85α subunit of PI 3-kinase. We first demonstrated p85α binding to CL6 CYT, and by mutational analysis showed that p85α was recruited to the same phosphorylated tyrosine residue present in the membrane-proximal ITIM. The classical recognition motif for the SH2 domains of p85α is a tyrosine-phosphorylated YMXM sequence (36). Therefore, p85α binding to CL6 CYT, like p85α binding to the hepatocyte growth factor and erythropoietin receptors (37, 38), involves SH2 domain-mediated recognition of a nonclassical sequence.

Other KIR CYT, including CL42, NKAT3, and NKAT4 CYT, also bound p85α to the same or lesser degrees as CL6 CYT. Subtle sequence differences in the region of the membrane-proximal ITIM between these CYT may explain differences in the ability to bind p85α and help define a consensus p85α recognition motif for KIR. In contrast, CL39 and KIR103AS CYT completely failed to bind p85α. Lack of binding with CL39 CYT can be explained on the basis that the single tyrosine of this tail is the penultimate residue of the receptor. Lack of binding with KIR103AS CYT was more surprising because in the region of the membrane-proximal ITIM, this CYT is identical to CL6 and NKAT4 CYT, which bind p85α strongly. Possibly, the divergent C-terminal sequence of KIR103AS CYT influences KIR CYT tertiary structure and prevents binding to p85α.

Regardless of structural considerations, it is interesting that an ability to bind or not bind p85α correlates with the function of receptors on NK and T cells. Thus, although p50 receptors such as CL39 are referred to here as KIR (based on extensive

**Fig. 5.** KIR-mediated PI 3-kinase-dependent activation of AKT. (A) and (B) Rested NK3.3 cells were not stimulated or were stimulated with combinations of GL183 plus GAM and PMA (1 ng/ml) plus Ionomycin (1 μM) (P/I) for 10 min at 37°C. In B the PI 3-kinase inhibitors wortmannin (0.2 μM) and LY294002 (10 μM) also were incubated with cells. Cells were lysed, AKT was immunoprecipitated, and AKT kinase activity was determined in *in vitro* kinase assays by using histone 2B as a substrate. The position of 32P-labeled histone 2B is shown. Relative scanning densitometry readings of phosphorylated histone 2B are indicated for each condition of stimulation.
homology of their extracellular domains to p58 receptors), these receptors in fact activate NK cell cytotoxicity (39). In fact, in most families of ITIM-bearing inhibitory receptors there exist noninhibitory or activating counterparts that are characterized, like p50 KIR, by the presence of a positively charged residue in their transmembrane domain (40). The KIR103AS receptor also contains a positively charged residue in its transmembrane domain, suggesting that it too may function as an activation receptor. In support of this, we have recently found that KIR103AS, in contrast to other KIR, enhances granule release from FcεRI-stimulated rat basophilic leukemia cells (unpublished observations).

PI 3-kinase is thought to function as a positive regulator of diverse cellular processes, including cell growth, cytokine production, and vesicle trafficking (18). Therefore, the coupling of inhibitory KIR to PI 3-kinase demonstrated here in both yeast and in NK cells suggests that these receptors may also convey positive signals. The kinds of positive cellular responses KIR may induce are unclear. However, the finding that KIR activation of PI 3-kinase leads to activation of the antia apoptotic kinase AKT suggests that one type of positive signaling event that KIR may be involved in is protection of NK cells from apoptotic death (19). This may be important in preventing NK cell suicide (41), for example, during NK cell recognition of nonthreatening class I MHC-bearing target cells.

Finally, it will be important to demonstrate that other classes of inhibitory receptor also recruit and activate PI 3-kinase. Indeed, preliminary evidence indicates that at least one other inhibitory receptor, CD22, recruits PI 3-kinase to its CYT (42). An ability to recruit and activate PI 3-kinase, therefore, may be a general feature of ITIM-bearing inhibitory receptors expressed on hematopoietic cells.

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